

## RESEARCH ARTICLE

## TECHNIQUES AND RESOURCES

# A transgenic quail model that enables dynamic imaging of amniote embryogenesis

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## ABSTRACT

Embryogenesis is the coordinated assembly of tissues during morphogenesis through changes in individual cell behaviors and collective cell movements. Dynamic imaging, combined with quantitative analysis, is ideal for investigating fundamental questions in developmental biology involving cellular differentiation, growth control and morphogenesis. However, a reliable amniote model system that is amenable to the rigors of extended, high-resolution imaging and cell tracking has been lacking. To address this shortcoming, we produced a novel transgenic quail that ubiquitously expresses nuclear localized monomer cherry fluorescent protein (chFP). We characterize the expression pattern of chFP and provide concrete examples of how Tg(PGK1:H2B-chFP) quail can be used to dynamically image and analyze key morphogenetic events during embryonic stages X to 11.

**KEY WORDS:** Confocal microscopy, Quail embryo, Live imaging

## INTRODUCTION

It is easy to overlook that the developing embryo – biochemically, histologically and molecularly detailed for over a century in static splendor – is undergoing constant change. Embryos are composed of many different progenitor and differentiated cells, arranged in complex 3D relationships, continually undergoing both unique and collective movements. Morphogenesis, the process whereby cells reorganize in 3D space to generate tissues and organs, results from a continual series of changes in cell shape, polarity, proliferation and movement. Imaging fixed embryos at a series of distinct developmental time points, although highly valuable, might not provide a complete depiction of these developmental processes over time and may inevitably miss important and transient changes. Capturing these changes in real time as the living embryo develops can add significantly to our understanding of complex cellular processes.

Dynamic imaging of embryo development has usually been carried out in lower vertebrates and invertebrates, but rarely in amniotes such as rodents owing to the difficulties of accessing the living embryos. Avians have long been a favorite model organism of classical embryologists because the embryos are highly accessible

to experimental manipulations and can be cultured artificially outside of the egg (Stern, 2005). In addition, the avian embryo is essentially a flat disc during the early events of development, such as gastrulation, axis patterning and somitogenesis. These same traits make the avian embryo amenable to dynamic imaging and the technique has long been utilized by embryologists (Bortier et al., 1996; Kulesa and Fraser, 2011). Recent innovations in bioimaging techniques and technologies facilitate investigation of the dynamic processes of avian embryogenesis at unprecedented spatial and temporal resolution. Specialized *ex ovo* culture techniques and computer-controlled microscope-mounted environmental chambers help us to meet the essential requirement of time-lapse imaging: that the embryo continues to live and function normally throughout the course of image acquisition.

Real-time 4D (*xyz*) imaging uniquely permits the behavioral history of individual and populations of live cells to be recorded in their natural environment with subcellular resolution (Bower et al., 2011). Performing time-lapse imaging after transiently labeling a small population of cells with vital markers by the injection of dyes, virus or the electroporation of plasmid DNA has been enormously successful in fate mapping and cell lineage studies in avian embryos (Bhattacharyya et al., 2008; Krull, 2004; Voiculescu et al., 2008). Despite their great utility, these techniques do not allow for long-term cell fate tracking of a large number of cells, as the reporter molecules are typically expressed in only a small proportion of the targeted cells and the label may be diluted out during cell division. The ability of an exogenous promoter to drive consistent, heritable, long-term expression of a vital marker, such as a fluorescent protein, in all of the cells in the embryo is the main strength of a transgenic avian as a model imaging system.

Transgenic avians that express fluorescent reporters constitutively, or in a cell-specific manner, allow for the direct imaging of a large number of cellular behaviors including tissue assembly and differentiation. Over recent years a number of transgenic chicken lines have been produced using eGFP as the marker for the transgene driven by strong ubiquitous promoters such as CMV, RSV, PGK and CAG (McGrew et al., 2004, 2008; Chapman et al., 2005; Koo et al., 2004; Motosono et al., 2010; Macdonald et al., 2012; Park and Han, 2012). In addition, transgenic quail lines using cell-specific promoters have allowed the dynamic imaging of particular tissue types, including endothelial cells (Sato et al., 2010) and neurons (Scott and Lois, 2005; Seidl et al., 2013). Targeting the fluorescent molecule as a fusion protein to a particular organelle, such as the cell membrane (Rozbicki et al., 2015), has led to additional insights into cellular movement and shape changes during primitive streak formation. In particular, expressing the fluorescent protein in the nucleosomes of chromatin within the cell nucleus has helped to facilitate the automated cell tracking of a large number of cells simultaneously (Sato et al., 2010). Building on the strengths of

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these previous transgenic avians, we set out to design a transgenic quail model that would be uniquely useful for the dynamic imaging and quantitative cell tracking analysis of early embryogenesis.

Here we report the development and characterization of this novel transgenic quail that ubiquitously expresses a nuclear localized red fluorescent protein, monomer Cherry (chFP), along with the imaging approaches used to record the dynamic events of early amniote embryogenesis. This transgenic line affords excellent visual access to the many cellular behaviors and morphogenetic events of embryogenesis beginning in the unincubated blastoderm (stage X) and continuing through developmental stage 11. We detail the use of several dynamic microscopy approaches, including multispectral 4D imaging and quantitative analysis, to demonstrate the utility of fluorescent transgenic quail embryos in visualizing several complex morphogenetic events during early avian embryogenesis, including gastrulation, head fold process formation, head patterning and dorsal aortae formation.

## RESULTS

### Generation of a PGK1:H2B-chFP transgenic quail line

We have engineered a novel transgenic quail line that ubiquitously expresses histone 2B-mCherry fluorescent protein (H2B-chFP) under the control of the human *PGK1* promoter, which controls transcription of the glycolytic enzyme phosphoglycerate kinase 1. We used a self-inactivating, replication-defective HIV-based lentivirus to infect and deliver the *PGK1:H2B-chFP* transgene (Fig. 1A) into the genome of quail stage X epiblastic germ cells. At 8 weeks post hatch, chimeric founders were bred with age-matched wild-type quail to obtain germline transmission of the *PGK1:H2B-chFP* transgene. Given the ubiquitous nature of the glycolytic pathway, we assumed that the Tg(PGK1:H2B-chFP)-positive offspring would express the transgene in the chorioallantoic membrane (CAM) that remains in the eggshell after hatching. This was indeed the case and facilitated screening for transgenic founder lines using an epifluorescence stereomicroscope (supplementary material Fig. S1).

Southern blot analysis of genomic DNA obtained from the CAM tissue of putative transgenic quail offspring confirmed the generation of three independent transgenic quail lines (Q1–3) that each contained a single copy of the transgene (supplementary material Fig. S2). All three Tg(PGK1:H2B-chFP) quail lines ubiquitously expressed H2B-chFP at roughly similar levels, as determined in images collected by epifluorescence microscopy (data not shown). We chose line Tg(PGK1:H2B-chFP)1R1a (Q1) to characterize further because the chFP was more highly expressed than in the other two lines allowing the minimal amount of laser power to be used during live imaging, thus limiting any phototoxicity effects. H2B-chFP is well tolerated by quail, as evidenced by the stable propagation of the transgenic line for over ten generations.

### Tg(PGK1:H2B-chFP) quail ubiquitously express H2B-chFP during developmental stages X–11

To confirm that Tg(PGK1:H2B-chFP) embryos ubiquitously express H2B-chFP from developmental stages X–11 (Ainsworth et al., 2010; Eyal-Giladi and Kochav, 1976; Hamburger and Hamilton, 1951), we fixed the embryos at various stages, counterstained the embryos with DAPI to label all cell nuclei, and imaged the embryos in 3D (*xyz*) by confocal and two-photon microscopy. Representative images of whole-mount embryos and high-resolution sections from stages X, 2, 5, 8 and 11 are shown (Fig. 1; supplementary material Figs S3–S6). We identified

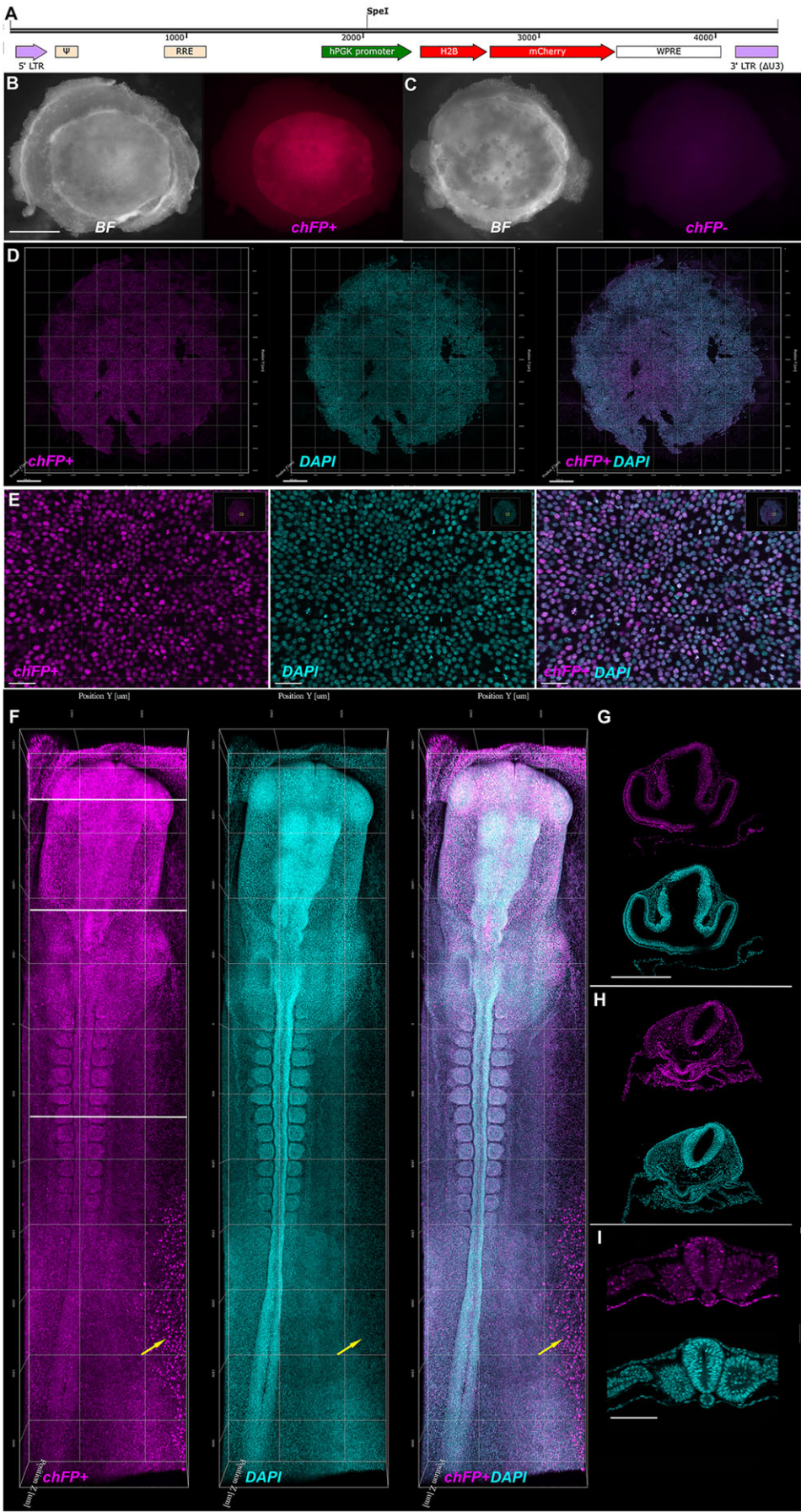
and counted chFP<sup>+</sup>/DAPI<sup>+</sup> nuclei both manually and with cell counting software (Imaris). Few if any cell nuclei were found that were DAPI<sup>+</sup>/chFP<sup>–</sup> in the embryonic cells of developmental stages X to 11. Original confocal microscopy image stacks of fixed Stage X, HH5 and HH11 Tg(PGK1:H2B-chFP) embryos counterstained with DAPI are available from the Dryad Digital Repository at <http://dx.doi.org/10.5061/dryad.74bk6>.

### Heterogeneity in H2B-chFP expression linked to cell proliferation rates

We noticed that different cells and tissues within embryos of the Tg(PGK1:H2B-chFP) lines display distinct yet reproducible H2B-chFP fluorescence levels from developmental stages X–11 (Fig. 1B–G; supplementary material Figs S4–S6). To determine if the heterogeneous intensities of H2B-chFP fluorescence within cell nuclei correlate with DNA content as the cell passes through cell cycle stages G1–S–G2, we examined the Tg(PGK1:H2B-chFP) embryos stained with DAPI at various developmental stages. DAPI interacts stoichiometrically with AT-rich regions of DNA and thus can be used as a fluorescent gauge for the DNA content within cells in various stages of the cell cycle (Crissman and Hirons, 1994). Contrary to our expectations, the chFP and DAPI relative fluorescence intensities did not correlate within the nuclei of Tg(PGK1:H2B-chFP) embryos (arrows in Fig. 1F; supplementary material Fig. S3D and Fig. S4E,F), suggesting that heterogeneity in H2B-chFP fluorescence intensity does not simply result from the expected changes in DNA content due to DNA replication.

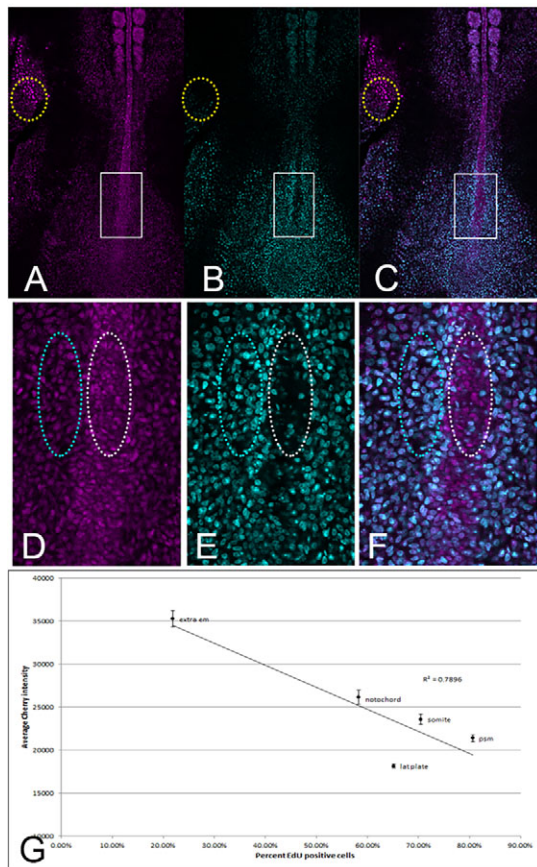
We hypothesized that the tissue-specific differences in chFP relative fluorescence could be due to differences in cell cycle length. Indeed, in transgenic mouse models it has been observed that fluorescent proteins linked to H2B tend to accumulate in the nuclei of cells that have a slower proliferation rate (Tumbar et al., 2004). To test this hypothesis we quantified the proliferation rate of embryonic cells in different tissues using a 6 h EdU incorporation, and compared the cellular proliferation rates with the chFP intensities of cells in each tissue. EdU is a thymidine analog that incorporates into the DNA of proliferating cells during DNA replication and can be detected by coupling to small fluorescent azides that easily penetrate the whole-mount embryonic tissue (Salic and Mitchison, 2008). We first noted that cells within the posterior tip of the notochord, which have a low rate of EdU incorporation, exhibit higher relative fluorescence than the adjacent lateral plate mesoderm or the presomitic mesoderm (PSM), which have a higher rate of EdU incorporation (Fig. 2A–F). In general, we have observed a robust inverse correlation between EdU incorporation rates and H2B-chFP relative fluorescence level averages for various cells and tissues (Fig. 2A–G;  $n=3$  experiments). For instance, cells in tissues with low rates of EdU incorporation, such as the extra-embryonic endoderm where 20% of the cells incorporated EdU within 6 h, exhibit a relatively high H2B-chFP expression average of ~35,000 relative fluorescence units (RFUs) (Fig. 2A–C, yellow ovals, 2G). By contrast, a high rate of EdU incorporation corresponded to low levels of H2B-chFP. For instance, in the PSM 82% of the cells incorporated EdU within 6 h and had an average H2B-chFP intensity of ~21,000 RFUs (Fig. 2D–F, blue ovals, 2G). We defined a statistically significant inverse linear correlation between rates of cell proliferation and average chFP intensities for several embryonic tissue types (Fig. 2G;  $R^2=0.79$ ,  $P=0.044$ ,  $n=5$ ). These results suggest that heterogeneity in H2B-chFP expression is linked to tissue proliferation rate. Therefore, Tg(PGK1:H2B-chFP) transgenic quail embryos can be used as a tissue proliferation reporter to dynamically determine the growth potential of embryonic territories.





**Fig. 1. Characterization of developmental stage X and 11 Tg(PGK1:H2B-chFP) quail.** (A) Schematic representation of the PGK1:H2B-chFP lentivector following chromosomal integration. The length of the proviral sequence from the 5' LTR to the 3' LTR is 4352 bp. The *SpeI* restriction site used to digest the genomic DNA for Southern blotting analysis is indicated. LTR, long terminal repeat; psi, packaging signal; RRE, Rev-response element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. (B,C) Stereomicroscopy images of developmental stage X (unincubated) Tg(PGK1:H2B-chFP) (B) and wild-type (C) embryos. The embryos have been isolated away from the egg yolk. BF, brightfield. (D,E) Confocal images for chFP<sup>+</sup>, DAPI, and chFP<sup>+</sup>/DAPI overlay of developmental stage X Tg(PGK1:H2B-chFP) whole-mount quail blastoderm (D) and higher magnification images to confirm ubiquitous and heterogeneous chFP expression (E). Variable chFP expression can be seen from cell to cell in the epiblast monolayer at stage X that does not directly correlate with DAPI fluorescence intensity. (F) Confocal images for chFP<sup>+</sup>, DAPI, and chFP<sup>+</sup>/DAPI overlay of developmental stage 11 Tg(PGK1:H2B-chFP) whole-mount quail embryos. Some extra-embryonic cells consistently display very high levels of chFP fluorescence (yellow arrows). Dorsal perspective. Grid scale marked every 500 μm along xy axes. (G-I) Transverse Vibratome section images for chFP<sup>+</sup> and DAPI at approximately the A-P region indicated by the white lines in F confirm ubiquitous but heterogeneous chFP expression. Scale bars: 1 mm in B,C; 500 μm in D,G,H; 200 μm in I; 50 μm in E.





**Fig. 2. H2B-chFP levels correlate with cell cycle time in embryonic tissues.** (A–F) Confocal z-slices of EdU staining (6 h incorporation; cyan) and chFP fluorescence (magenta) in the posterior part of a stage 10 Tg(PGK1:H2B-chFP) quail embryo. (A,D) chFP signal, (B,E) EdU signal, (C,F) overlay. The boxed areas in A–C are magnified in D–F. Yellow ovals show low EdU/high chFP levels in extra-embryonic endoderm. Blue ovals show high EdU incorporation in the PSM and corresponding low chFP intensity. White ovals show low EdU incorporation in the posterior tip of the notochord and corresponding high chFP level. (G) The average cellular chFP intensity for different regions of the embryo compared with the percentage of EdU incorporation, showing significant linear correlation ( $R^2=0.79$ ,  $P=0.044$ ,  $n=5$ ); errors bars indicate s.e.m. Extra em, extra-embryonic endoderm; lat plate, lateral plate; psm, presomitic mesoderm.

### Dynamic analysis of morphogenesis in living embryos

Supplementary material Movie 2 illustrates the use of 4D ( $xyz$ ) confocal laser microscopy to time-lapse record a Tg(PGK1:H2B-chFP) quail embryo maturing from developmental stages 3–8 with a montage of images taken every  $\sim 15$  min for 16.5 h. The gastrulating embryo elongates along the anterior-posterior (A–P) axis as converging epiblast cells intercalate along the midline to form the primitive streak (PS) ( $\sim 3:00:00$  time point). The head fold process begins ( $\sim 5:30:00$ ) concomitant with continued A–P elongation along the midline PS. Somites 1 begin to form bilateral and adjacent to the PS ( $\sim 8:30:00$ ) and continue until the movie ends at the 4-somite stage ( $16:30:00$ ).

4D imaging permits individual cells to be tracked over time and z-layer. For example, supplementary material Movie 1 focuses on the head fold process apparent within supplementary material Movie 2 with higher spatial resolution (Fig. 3A). Supplementary material Movie 1 is a maximum intensity projection of the head fold process showing elongation along the A–P axis as converging epiblast cells intercalate along the midline, thrusting the PS and adjacent tissue

anteriorly. At this point during development, the flat trilaminar embryonic disk begins to form into a 3D embryo. Three distinct morphogenetic events can be seen occurring concurrently in supplementary material Movie 1A: (1) the anteriormost tissue of the embryo folds ventrally as a distinct tissue layer and descends in the posterior direction; (2) as the ventral tissue moves posteriorly, the adjacent lateral tissue simultaneously folds toward the midline to form the anterior intestinal portal (AIP); and (3) the PS and node regress posteriorly, while in the anterior part of the elongating embryo the notochord and neural tube form and extend along the A–P axis. They are displaced dorsally as the dorsal-ventral (D–V) axis thickens.

Cell tracking software was used to quantitatively analyze and visualize the movement of individual cells. White ‘dragon tails’ showing the direction and speed of migration of individual tracked cells for the previous four time points (60 min) were superimposed upon the image set of the embryos (supplementary material Movie 1B). Supplementary material Movie 2C,D similarly exhibit the spatiotemporal location and speed of migrating embryonic cells and are color-coded to better visualize movement along the y-axis for the previous four time points (60 min) (Fig. 3B–D).

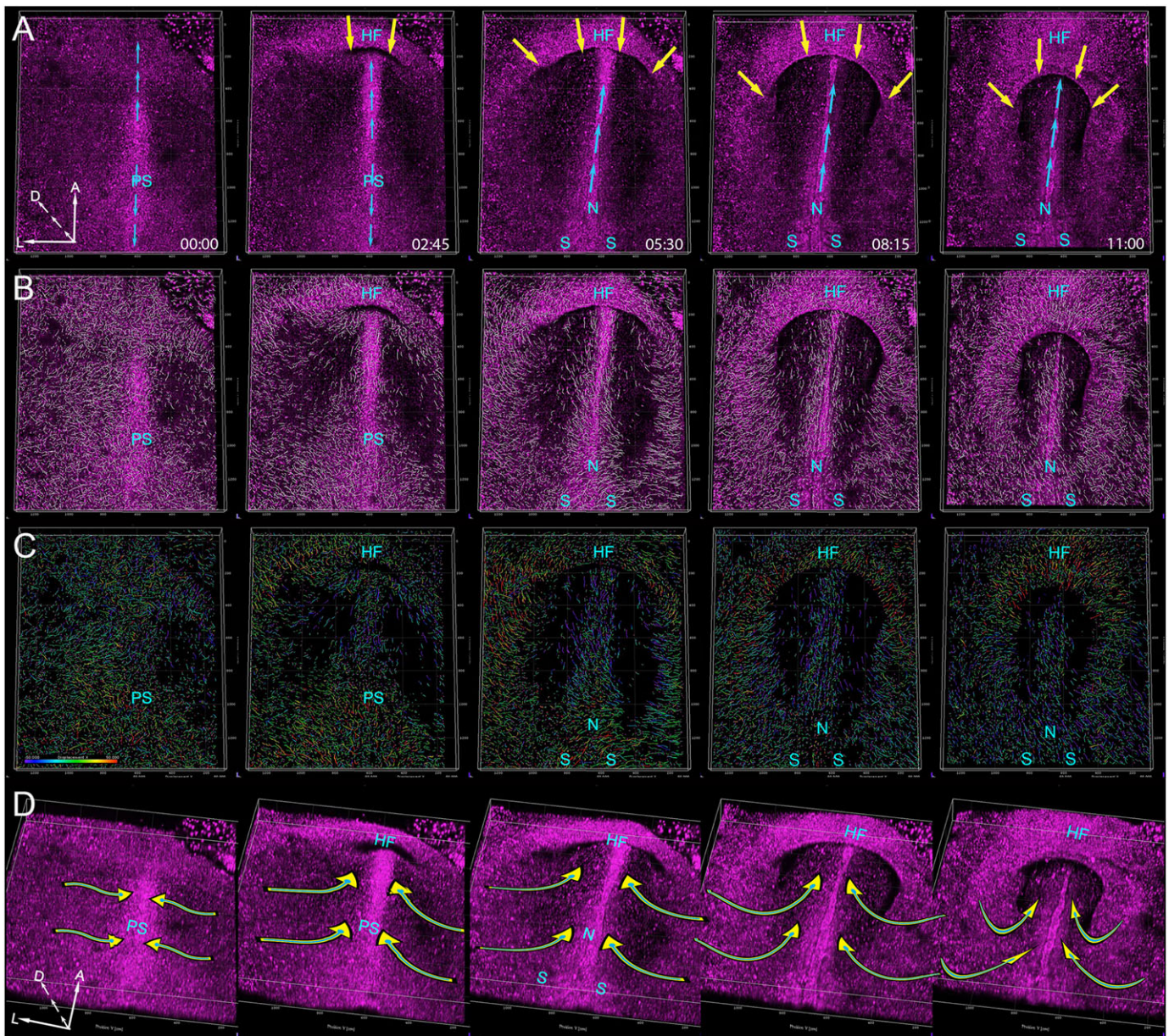
To acquire a global view of cell and tissue movements during embryogenesis, we also used wide-field epifluorescence imaging and a whole-yolk, *ex ovo* avian embryo culturing method (Czirok et al., 2002). This combination permits the embryo to be observed for up to 2.5 days because the low level of applied light does not harm the living embryo. Time-lapse epifluorescence microscopy was used to image a Tg(PGK1:H2B-chFP) quail embryo from developmental stages 3 to 11 with images taken every  $\sim 10$  min for 20 h (of 48 h) (supplementary material Movie 3).

Taken together, supplementary material Movies 1–3 indicate that living Tg(PGK1:H2B-chFP) quail embryos are able to withstand the rigors of long-term fluorescence video-microscopy during gastrulation, thus providing a novel model organism with which to dynamically analyze embryogenesis in amniotes with a spatiotemporal resolution that permits cell movements to be individually and collectively analyzed using statistical approaches that yield quantitative data.

### Cell proliferation and migration during head morphogenesis

Head development is a complex process that involves the assembly and integration of distinct cell and tissue types. The cranial neural folds fuse to form the presumptive ectoderm, neural tube and neural crest, while enveloped with an ectoderm layer that together will form the developing head. Cranial neural crest (CNC) cells and paraxial mesoderm in the middle of the embryo (Noden and Trainor, 2005) produce the craniofacial mesenchyme that differentiates into the cartilage, bone, cranial neurons, glia, and connective tissues of the face. In order to understand the cellular dynamics of early head formation, Tg(PGK1:H2B-chFP) quail embryos were video recorded from the dorsal perspective using 4D confocal microscopy from stages 8–10 (supplementary material Movies 4–6). High-resolution static confocal microscopy confirms that the ectoderm- and mesoderm-derived cells within the developing head region are chFP<sup>+</sup> (Fig. 1D,E and Fig. 3; supplementary material Fig. S5). The 4D rendering of Tg(PGK1:H2B-chFP) head formation shows the stage 8 embryo elongating along the A–P axis (Fig. 4; supplementary material Movies 1 and 4). It is difficult to distinguish the individual and collective cell movements in the more ventral z-sections because all chFP<sup>+</sup> cells are similarly excited with the 561 nm laser, causing a cherry emission haze from out-of-focus cells. To obtain better cell and tissue resolution, we analyzed individual and action-grouped z-sections (supplementary material





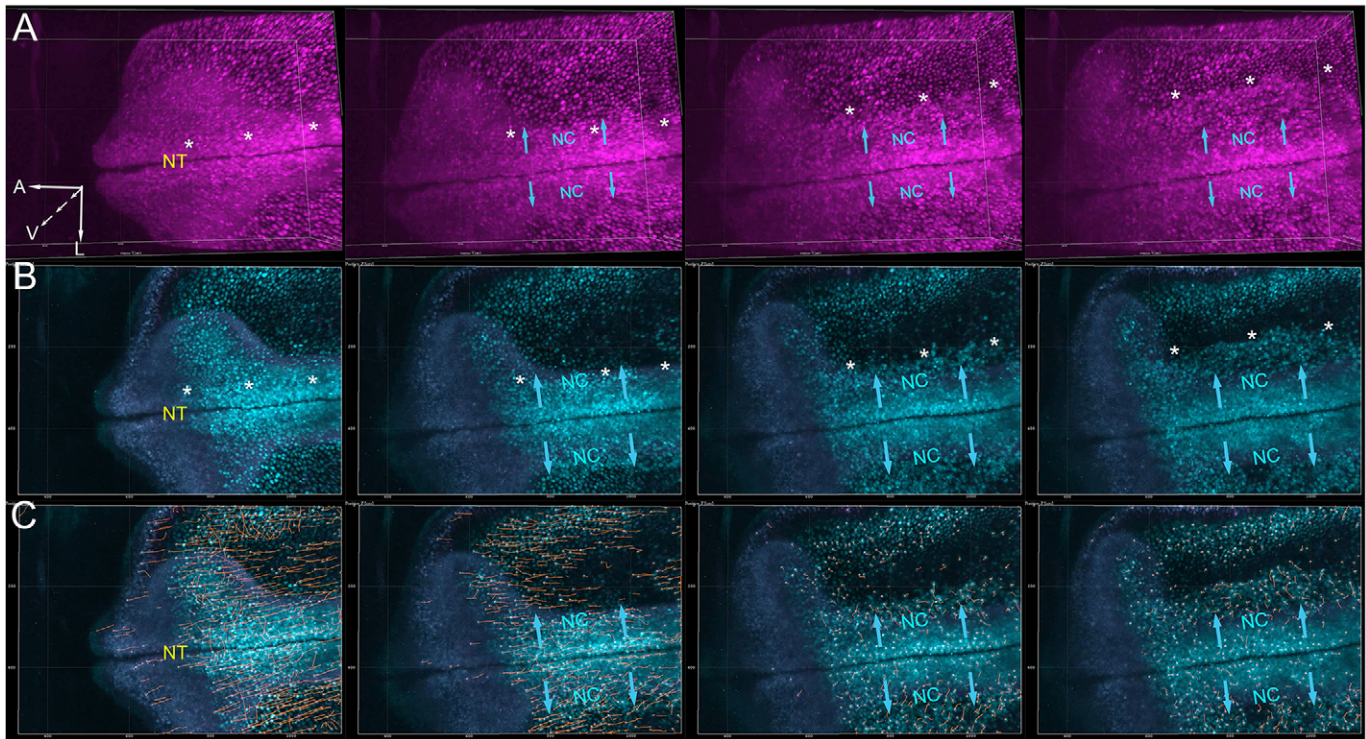
**Fig. 3. Representative images from time-lapse microscopy of a Tg(PGK1:H2B-chFP) embryo showing cell and tissue movements of the head fold process.** (A) Five representative images from supplementary material Movie 1A of the head fold process in the gastrulating embryo, showing elongation along the A-P axis as converging epiblast cells intercalate along the midline, thrusting the PS and adjacent tissue anteriorly until they fold ventrally and then descend along the posterior direction. As the ventral tissue moves posteriorly, adjacent lateral tissue simultaneously folds toward the midline to form the AIP. Cyan sets of three arrows highlight dorsal tissue movement along the midline in the A-P directions. Yellow arrows highlight ventral tissue movements of the head fold process and resulting AIP formation in the ventral and medial directions. (B) Five representative images from supplementary material Movie 1B. Tracking cells to show the direction and speed of migration. The cell tracks, shown as white 'dragon tails' represent the location of a cell for the previous four time points (60 min). (C) Five representative images from supplementary material Movie 1C,D. Tracking cells to indicate movement along the y-axis, direction and speed of migration. The cell tracks are color-coded 'dragon tails' that represent the location and movement of a cell along the y-axis ( $-50$  anterior to  $50$  posterior; displacement bar bottom left) for the previous four time points (60 min). (D) Images from supplementary material Movie 1E. These images, which originally are shown in supplementary material Movie 1A, have been re-oriented and zoomed to better visualize the apparent movements of cells and tissue as the embryo changes shape along the D-V axis. The anterior end is oriented away from the viewer and the central axis is tipped slightly to the right. This orientation gives the viewer a perspective of looking into the forming AIP and permits the medio-lateral tissue movements concomitant with AIP formation to be better visualized. The yellow and cyan arrows highlight the predominant movement of the majority of the cells converging toward the midline as the embryo folds to form the coelomic cavity. Grid scale is marked every  $500\ \mu\text{m}$  along xy axes. The representative images in A-C are at identical time points, as indicated bottom right (h:min) and correspond to the time points of supplementary material Movie 1. A, anterior; L, lateral; D, dorsal; PS, primitive streak; HF, head fold; N, notochord; S, somite.

Movie 5), which were then color-coded for further visual discrimination (supplementary material Movie 6).

The single layer of ectoderm cells that envelopes the dorsal head region of the embryo was collected in layers z1-z2 along the midline

and in additional z-layers on the lateral periphery, since the embryonic head is curved (supplementary material Movie 5A). The ectoderm cells ( $\sim 42$  cells/ $100\ \mu\text{m}^2$ ) show minimal autonomous cell movements and few neighbor-neighbor positional changes,





**Fig. 4. Distinct cell behaviors in different z-layers during head formation.** (A) Representative images derived from supplementary material Movie 4 of a 4D rendering of the forming head region of a Tg(PGK1:H2B-chFP) embryo at ~105 min intervals. In the first frame, the embryo is elongating along the A-P axis as it moves to the left side of the image. As the anterior movement subsides, neural crest cells begin to delaminate from the NT and migrate bilaterally and ventrally along the inner side of the non-neural ectoderm (leading edge of migrating neural crest cells is marked with white asterisks and direction of migration is marked by cyan arrows) as seen in the next three frames. (B) z-layers were distinctly pseudo-colored to better visualize the relative movements of chFP<sup>+</sup> cells and tissues in different z-layers, (z3-7 in cyan and z10-14 in magenta). Neural crest cells egress from the NT and bilateral migration (cyan arrows) begins, which can be viewed in supplementary material Movie 6B. (C) Tracked cells are marked by orange 'dragon tails' to help visualize the speed and direction of individual cell movements, which can be viewed in supplementary material Movie 6C. Dorsal view. Grid scale is marked every 200  $\mu$ m along xy axes. A, anterior; L, lateral; V, ventral; NT, neural tube; NC, neural crest.

except after mitosis, thus acting and moving collectively as an epithelial sheet. The epithelial cells are actively dividing, but there is no apparent coordination in their cell cycle or orientation of cell division (supplementary material Movie 5A,B).

Confocal layers z3-z7 visually captured the mesencephalic neural crest cells as they delaminate from the neural tube (NT) via epithelial-mesenchymal transformations and migrate *en masse* bidirectionally and dorsolaterally along the curved basal side of the non-neural ectoderm cells into anatomical territories rich in mesodermal cells (supplementary material Movie 5C,D and Movie 6A-D; Fig. 4B). The CNC cells move ~200  $\mu$ m in 10 h, or ~20  $\mu$ m/h. The migrating CNC cells show limited cell proliferation, possibly tempered by their strong lateral migration. The head mesenchyme cells, which can be seen ventral and lateral to the NT, move randomly and independently of one another, while occasionally undergoing cell divisions within the cavity (supplementary material Movie 5E). Finally, the entire NT extends ventrally beyond the ideal resolving power of the laser microscope (~75  $\mu$ m). The cell density of the NT is too high to dynamically resolve the individual cells with the microscope settings used. However, it is possible to see NT morphogenesis at the tissue level (supplementary material Movie 5E and Movie 6). The confocal layer z14 movie segment focuses on the NT as it moves anterior and presses against the anteriormost ectoderm cells, flattening and then bifurcating laterally to form the optic vesicles.

Thus, the distinct movements of the various cell populations involved in stage 8-10 head formation can be readily visualized, resolved and analyzed within multiple independent z-layers. The

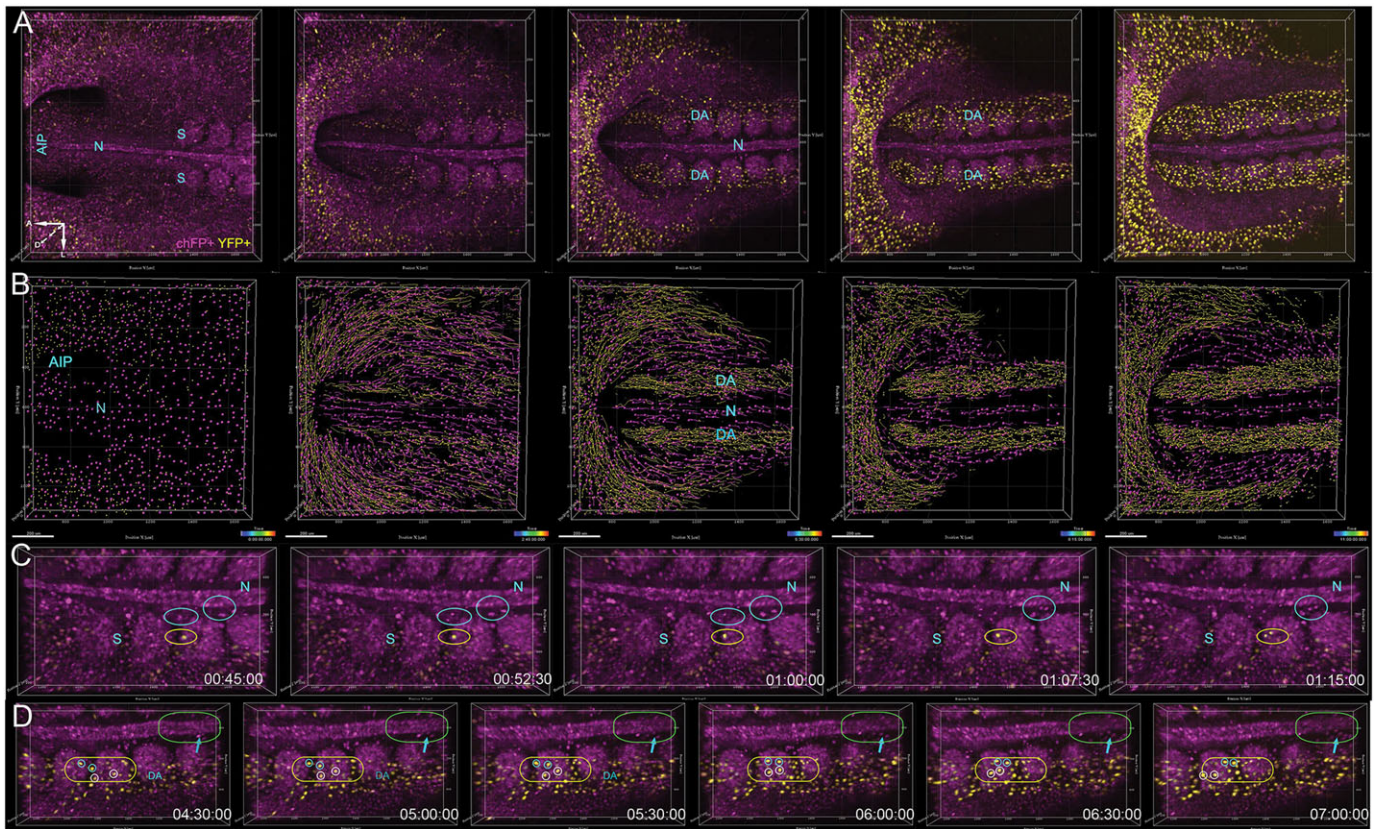
directed migration of the CNC cells stands in contrast to the ballooning expansion of the embryo-enveloping ectoderm, the sparse and randomly moving head mesenchyme, and the anterior surging NT.

#### Multispectral dynamic analysis of aortic vasculogenesis

Vasculogenesis, the *de novo* assembly of blood vessels, involves changes in cell proliferation, differentiation and morphogenesis. The Tg(PGK1:H2B-chFP) and Tg(TIE1:H2B-eYFP) quail lines were interbred to produce stable Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) transgenic lines to study the events of vascular development. The *TIE1:H2B-eYFP* transgene marks endothelial cells (ECs) with nuclear localized eYFP (Sato et al., 2010). The Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) double-transgenic quail embryos permit all cells, including putative angioblasts (defined here as having chFP<sup>+</sup>/YFP<sup>-</sup> nuclei that soon become chFP<sup>+</sup>/YFP<sup>+</sup> nuclei), to be tracked by multispectral 4D imaging in the red channel and the ECs and endocardial cells to be separately identified and tracked in the yellow channel.

As a proof of concept, we dynamically imaged dorsal aortae assembly using Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) embryos at stages 6-11. A representative time-lapse movie shows the assembly by vasculogenesis of adjacent dorsal aortae (supplementary material Movie 7). Initially, mesoderm cells proliferate and differentiate into angioblasts (supplementary material Movie 7; Fig. 5). Similarly, the putative angioblasts appear to proliferate and differentiate into ECs, interact and assemble into blood vessels that soon form lumens to permit blood flow and vascular networks to enable circulation





**Fig. 5. Representative images of multispectral 4D imaging of a Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) embryo shows assembly of dorsal aortae within the developing ventral trunk region.** (A) Representative image sequence from supplementary material Movie 7 showing dorsal aortae formation in a Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) double-transgenic quail embryo with chFP+ cell nuclei (magenta) and YFP+ EC nuclei (yellow). (B) chFP+/YFP+ cell tracking data from image sets used to generate supplementary material Movie 8 of a Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) embryo. Scale bars: 150  $\mu$ m; grid scales mark every 200  $\mu$ m along xy axes. (C) Higher magnification images from supplementary material Movie 10B demonstrating active cell proliferation during dorsal aorta formation. The series of images cover from time point 00:45 to 01:15:00 hrs in 07:30 min intervals. Numerous cell divisions occur throughout supplementary material Movie 10. Some of these divisions are highlighted within the cyan ellipses (dividing chFP+ cells), while a dividing YFP+ EC is highlighted within the yellow circle. (D) Higher magnification images from supplementary material Movie 10 illustrating differential cell and whole tissue movements during dorsal aorta and notochord formation. The individual images cover time points 04:30:00 to 07:00:00 hrs in 30 min intervals. Cells within the stationary region of interest (ROI, yellow ellipse), which is drawn over the dorsal aorta (DA), show opposing cell movements. Putative angioblasts and ECs within the forming dorsal aorta (white circles) appear to move anteriorly with the majority of their neighboring aortic cells whereas some adjacent aortic cells (cyan circles) continue to move randomly or in a posterior direction. Likewise, within the green ROI, which incorporates the notochord and presumptive endoderm, there is a very bright chFP+ cell which begins at the cyan arrow reference point at time 04:30:00 then moves anterior at  $\sim 100$   $\mu$ m/h (similar to the somites, dorsal aorta, and adjacent tissue) while seven to ten chFP+ presumptive endoderm cells located just above the arrow remain relatively stationary. In contrast to the differential movement of individual cells, the entire notochord tends to move steadily in a posterior direction along with the majority of the adjacent cells. Grid scales marked every 200  $\mu$ m along xy axes; time scale in lower right corner; TIE1:H2B-eYFP+ ECs are pseudo-colored yellow; PGK1:H2B-chFP+ cells are pseudo-colored magenta; Time scale is in h:min:s. A, anterior; L, lateral; D, dorsal; AIP, anterior intestinal portal; N, notochord; S, somite; DA, dorsal aortae.

(supplementary material Movies 7–9). Putative angioblasts (chFP+/YFP-) can be seen interacting in the extra-embryonic vascular plexus as they proliferate, differentiate into ECs (chFP+/YFP+) within minutes of one another, and migrate to self-assemble the primary vascular plexus (supplementary material Movies 7–10). The aortic ECs appear to move quasi-collectively, since they are seen changing their neighbor-neighbor relations, yet are moving *en masse* in the same anterior direction (supplementary material Movie 10). The metaphase plate of dividing aortic ECs shows no obvious preferred orientation, which is likely to account for the concurrent increase in length and girth of the aortae at these developmental stages (Zeng et al., 2007). ECs from the adjacent vascular plexus can be seen streaming medially into the ventral half of the dorsal aortae, and toward the forming heart atria just above the descending AIP. It is readily apparent that the forming dorsal aortae, notochord, vascular plexus and somites are independently moving in the anterior direction at the region of the trunk that we are viewing as the

embryo elongates along the A-P axis (supplementary material Movies 7–9). However, we appreciate that the observed anterior movement is transient and stalls or changes direction as the embryo matures (supplementary material Movies 1 and 2). The example of dorsal aortae formation as visualized by multispectral 4D imaging of Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) embryos shows the seamless interplay of cells displaying distinct and overlapping behaviors that drive transient tissue assembly processes.

## DISCUSSION

### Tg(PGK1:H2B-chFP) quail permit dynamic investigations of morphogenetic processes

Many tools in developmental biology acquire static information to investigate the transient processes of the living embryo. It is our contention that to understand the actual physiological state of a cell or tissue it must be studied within the multicellular environment in which it evolved – the living embryo. Transgenic quail are amenable

to the rigors imposed by vital imaging and have sufficient optical transparency to record and study individual and collective cell behaviors *in vivo*. Multiple time-lapse movies demonstrate how various cells and tissues within living Tg(PGK1:H2B-chFP) embryos can be recorded and studied using vital imaging to examine transient developmental events, such as gastrulation (supplementary material Movies 1–3), neural development (supplementary material Movies 1–6) and vascular development (supplementary material Movies 7–10), with subcellular resolution. We showed for the first time in an amniote that the distinct movements of the three germ layers (endoderm, mesoderm and ectoderm) can be visualized, resolved and analyzed within multiple independent *z*-layers (supplementary material Movies 4–6). By crossing two independent transgenic quail lines we generated Tg(PGK1:H2B-chFP; TIE1:H2B-eYFP) double-transgenic embryos to highlight the potential of multispectral 4D imaging of living embryos.

The nuclear localized fluorescent protein of the Tg(PGK1:H2B-chFP) quail permits most cells to be recorded and distinguished from neighboring cells since the cytosol surrounding the nucleus is unlabeled. This, in turn, allows the use of automated image analysis software to segment and track embryonic cells *in vivo*, greatly facilitating the laborious task of quantitative analysis of hundreds to thousands of cells within whole embryos across multiple time points (Kanda et al., 1998; Sato et al., 2010).

#### **Tg(PGK1:H2B-chFP) acts as a dynamic reporter of cell proliferation**

Although the cellular expression of H2B-chFP is ubiquitous, the relative fluorescence intensity of chFP in individual cells and tissues is heterogeneous when viewed across time and space (Figs 1 and 2; supplementary material Figs S4–S6). Cells within the posterior tip of the notochord and extra-embryonic putative endoderm cells that incorporated EdU at a low rate also exhibited higher relative fluorescence than other tissues that proliferate more rapidly (Fig. 2). These data indicate that the Tg(PGK1:H2B-chFP) transgenic quail embryo is a reliable reporter of proliferation rate (Fig. 2). The observed cellular variability in H2B-chFP mean fluorescence intensities is a function of the transgene or its expressed cargo and not of transgene copy number or chromosomal integration site since each of three transgenic lines contain a single copy of the transgene integrated at a unique site within the genome (Feng et al., 2000). These results suggest that H2B-chFP accumulates in cells that proliferate slowly and is diluted out in cells that proliferate rapidly. Other studies have shown that transiently expressed H2B-GFP reporters accumulate and are diluted as a function of the cellular proliferation rate in mouse models, and thus provide a precise quantitative proliferation history of a cell or tissue (Foudi et al., 2009; Tumber et al., 2004).

It is well known that post-translational modifications of H2B regulate many processes within the nucleus, including transcription initiation and elongation, silencing and DNA repair (Weake and Workman, 2008). It might be that the expression of H2B-chFP is based on the regulation of the *PGK1* promoter region, the expressed transcript, or on the H2B-chFP protein itself due to chromatin interactions or post-translational modifications of histone 2B. Although resolving the exact molecular mechanism that underlies this observation will require additional studies, the heterogeneity of chFP expression in Tg(PGK1:H2B-chFP) quail should help us better understand how cell proliferation rates contribute to the differential growth of various tissues and regions during early embryogenesis (Ridenour et al., 2012; Sakaue-Sawano et al., 2008).

#### **Transgenic avians are providing new tools for a classical model system**

The Tg(PGK1:H2B-chFP) quail adds to an ever-growing list of transgenic lines generated over the past decade in quail (Sato and Lansford, 2013; Sato et al., 2010; Scott and Lois, 2005; Seidl et al., 2013), chicken (Balic et al., 2014; Chapman et al., 2005; McGrew et al., 2004; Motosono et al., 2010; Rozbicki et al., 2015) and songbird (Agate et al., 2009) that express reporter proteins in a ubiquitous or tissue-specific manner.

The traditional chick-quail chimera technique uses histological stains or immunohistochemistry to distinguish quail from chicken nucleoli in chimeric embryos for cell lineage studies (Le Douarin, 1973; Le Douarin and Barq, 1969; Le Douarin and Kalcheim, 1999). Since transgenic avians with ubiquitous promoters express their reporter proteins stably, their tissues are excellent candidates for classical ectopic grafting experiments, using fluorescent microscopy to track cell and tissue fates over long periods of time. For example, McGrew et al. (2008) grafted cells from their CAG:eGFP transgenic chicken line into wild-type hosts in order to elucidate the fates of separate groups of progenitor cells within the elongating tail bud. Recently, cloacal tissue from donor GFP transgenic chicken embryos transplanted into the hindlimb of wild-type chicken embryos demonstrated the conserved ability of some mesenchymal cells to respond to the cell signaling required for the formation of external genitalia (Tschopp et al., 2014). These studies clearly demonstrate the power of the transgenic chick-to-chick transplant technique. Likewise, because the H2B-chFP label is integral to all of the cells, does not spread to adjacent cells and will not disappear with cell proliferation, the Tg(PGK1:H2B-chFP) quail should allow transplanted cells to be easily distinguished from host cells and dynamically followed in chick-Tg quail or quail-Tg quail chimeras.

At the time of egg laying (stage EG.X), there are ~50,000 chFP<sup>+</sup> cells within the area pellucida of the Tg(PGK1:H2B-chFP) blastoderm (Fig. 1B–E; supplementary material Fig. S3). Stable chFP expression in these cells permits early cell movements of the developing blastula and gastrula (supplementary material Movie 2) to be dynamically studied, starting immediately in unincubated eggs and without the need for extrinsic labels such as injected vital fluorescent dyes or transfected fluorescent protein expression vectors, which typically take 1–10 h or more for the fluorescent proteins to be expressed at levels sufficient for dynamic imaging (Bower et al., 2011). Dye labeling approaches may not provide single-cell resolution and can fade over time due to dilution with cell division or from photobleaching during imaging (Bower et al., 2011; Clarke and Tickle, 1999). Electroporation into pre-stage 3 embryos is technically challenging as the embryos are very fragile and highly sensitive to the transfection procedure (Voiculescu et al., 2008). Electroporation, in particular, may introduce highly variable numbers of expression vectors per cell and thus their genetic cargo is expressed at highly variable levels, which greatly complicates image analysis (Momose et al., 1999; Nakamura et al., 2004). Despite these technical limitations, in cases in which the experimental question requires the labeling of a small population of cells with extrinsic markers, the Tg(PGK1:H2B-chFP) quail embryos should prove very useful. The ubiquitously labeled chFP<sup>+</sup> cells of the transgenic quail may provide the background tissue context in which the extrinsically labeled cells are moving and interacting.

#### **Technical considerations of the Tg(PGK1:H2B-chFP) model system**

The standard tiled multispectral 4D whole-embryo imaging experiment, as shown in supplementary material Movie 7, uses



confocal fluorescence microscopy to capture 50–200  $\mu\text{m}$  deep  $z$ -stacks every  $\sim 2$ –6 min for 6–48 h. Two-photon laser scanning microscopy (TPLSM) in theory offers deeper imaging, reduced photodamage from out-of-focus illumination, and the ability to image multiple labels in the same living specimen (Lansford et al., 2001). As a proof of concept, we used TPLSM to image through the entire ( $\sim 150$   $\mu\text{m}$  D–V) trunk region of a fixed stage 10 Tg(PGK1:H2B-chFP) embryo with subcellular  $z$ -resolution (supplementary material Fig. S6). We found that the Tg(PGK1:H2B-chFP) cells are best excited from 1040–1160 nm (data not shown), which agrees with previous reports (Drobizhev et al., 2011, 2014; Vadakkan et al., 2009). Unfortunately, high  $z$ -resolution image acquisition requires largely overlapping  $z$ -stacks and therefore results in longer laser exposure. Imaging with the high laser power necessary to excite chFP with TPLSM under high  $z$ -resolution conditions is highly deleterious to the living embryo during time-lapse imaging (data not shown), probably owing to photodamage (Drobizhev et al., 2011). We are confident that continued advances in confocal imaging platforms, such as highly efficient lasers coupled with more sensitive detectors, will reduce the laser power needed to excite and detect chFP, thus leading to greater embryo survival during dynamic TPLSM imaging sessions.

Because of its high-speed optical sectioning capabilities, light sheet microscopy can image large regions of a specimen with very high spatial and temporal resolution. This technique holds great promise for the dynamic imaging of actively developing transgenic avians. Recently, Rozbicki et al. (2015) used single-photon light sheet imaging to study the large-scale tissue flows associated with PS formation using a transgenic chicken ubiquitously expressing a membrane-linked GFP. Because all cell nuclei are fluorescent in Tg(PGK1:H2B-chFP) quail embryos, complex tracking analysis in both mesenchyme and epithelium is possible (supplementary material Movie 5). Therefore, when used in combination with new emerging microscopy techniques such as light sheet, the Tg(PGK1:H2B-chFP) quail will provide an excellent amniote model system for imaging the entire embryo through multiple early developmental stages.

## MATERIALS AND METHODS

### Ethics statement

All experimental methods and animal husbandry procedures were performed in accordance with the guidelines of the National Institutes of Health and with the approval of the Institutional Animal Care and Use Committee at the California Institute of Technology, the University of Southern California, and Children's Hospital Los Angeles.

### Plasmid construction and lentivirus production

The pLenti.hPGK1:H2B-chFP lentivector (Addgene plasmid # 51007) was derived from the pRRLsin.cPPT.PGKp.eGFP.wpre lentivector that contains the constitutively expressed human phosphoglycerate kinase 1 promoter (De Palma et al., 2003). The membrane localization tag of the pLenti.PGK1:membrane-mCherryFP lentivector was removed by *AgeI/BamHI* digestion and replaced by the *AgeI/BglII* human histone 2B region of pH2B-GFP (Kanda et al., 1998) to generate pLenti.PGK1:H2B-chFP (Fig. 1A) by standard DNA cloning techniques (Sambrook and Russell, 2001). VSV-g pseudotyped lentiviruses containing the pPGK1:H2B-chFP lentivector were produced and concentrated as previously described (Poynter et al., 2009; Sato et al., 2010). The H2B protein tag not only localizes chFP to the cell nucleus, but also within the nucleosomes of the chromatin (Kanda et al., 1998).

### Generation of Tg(PGK1:H2B-chFP) quail lines

Unincubated wild-type quail eggs were windowed and  $\sim 1$   $\mu\text{l}$  PGK1:H2B-chFP lentivirus ( $\sim 1 \times 10^9$  transforming units/ml) was injected into the subgerminal space of 415 stage X embryos, resealed and incubated until hatching. Ten embryos successfully hatched (2.4%). Upon sexual maturity, each of the ten G0 mosaic founders was bred to a separate wild-type mate,

with three producing stable transgenic Tg(PGK1:H2B-chFP) offspring. Transgenic hatchlings were identified using a fluorescent dissecting scope to screen for H2B-chFP<sup>+</sup> nuclei in cells of the CAM.

Southern blot analysis for the *chFP* transgene was carried out on genomic DNA isolated from the CAM using standard protocols (Sambrook and Russell, 2001) as described in the supplementary methods.

Stable breeding colonies for three independent Tg(PGK1:H2B-chFP)-expressing transgenic quail were established. These transgenic lines were named following nomenclature guidelines established for mice and rats: Tg(PGK1\_p:H2B-chFP)1Rla, Tg(PGK1\_p:H2B-chFP)2Rla, and Tg(PGK1\_p:H2B-chFP)3Rla (<http://www.informatics.jax.org/mgihome/nomen/gene.shtml>). The Tg(PGK1\_p:H2B-chFP)1Rla line has been maintained in our quail aviary for  $\sim 5$  years. Tg(PGK1\_p:H2B-chFP)1Rla was used for all of the studies presented.

### Embryonic staging

Quail embryos were assigned an embryonic developmental stage based on previously established criteria (Ainsworth et al., 2010; Eyal-Giladi and Kochav, 1976; Hamburger and Hamilton, 1951).

### Cell proliferation assay

The Click-it EdU assay (Invitrogen) was used to assay *in vivo* cell proliferation according to manufacturer's recommendations; for details, see the supplementary methods. The percentage of EdU-positive cells and level of chFP signals were quantified using the spot function of Imaris (Bitplane) software and analyzed as indicated in the supplementary methods.

### Microscopy and imaging

Wild-type and Tg(PGK1\_p:H2B-chFP)1Rla or Tg(PGK1\_p:H2B-chFP)1Rla; (TIE1\_p:H2B-eYFP)2Rla quail embryos were incubated at 37°C in a humidified incubator to the desired embryonic stages. Embryos were prepared for static whole-mount imaging, Vibratome sectioning and dynamic imaging as detailed in the supplementary methods. Imaging metadata and details of image analysis, including 3D imaging by two-photon microscopy and 4D imaging for time-lapse experiments, are provided in the supplementary methods.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceived and designed the experiments: D.H., B.B. and R.L. Performed the experiments: D.H., B.B., A.W., J.Y., M.F. and R.L. Analyzed the data: D.H., B.B., S.E.F. and R.L. Wrote the paper: D.H., B.B. and R.L.

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### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.121392/-/DC1>

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